INFLUENCE OF PARENTAL SWIMMING STAMINA ON THE CARDIAC AND METABOLIC PERFORMANCE OF LARVAL ZEBRAFISH (*Danio rerio*)

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Superior swimming stamina in adult fish is presumably passed on to their offspring, but the ontogeny of the appearance of superior stamina and the requisite enhanced cardio-respiratory support for locomotion in larval fishes has not been determined. Is the expression of the suite of parental traits enabling superior swimming stamina in their offspring dependent upon their achieving juvenile/adult morphology, or does it appear earlier in their larvae? To answer this, adults were classified into three groups based on swimming stamina, followed by measurement of length, mass, and width. Larval offspring from the two parental groups -high stamina larvae (HSL) and low stamina larvae (LSL)- were reared at 27°C in aerated water (21% O_2). Routine and active heart rate (fH,r and fH,a, respectively), routine and active mass specific oxygen consumption ($\dot{M}O_2$,r/$\dot{M}O_2$,a) were recorded through 21dpf, and cost of transport (COT) and factorial aerobic scope were derived from oxygen consumption measurements. The fH,r at 2dpf of LSL was 164 ± 1 b·min^{-1}, compared to only 125 ± 2 b·min^{-1} for HSL. fH,r subsequently peaked at 203 ± 1 b·min^{-1} at 5dpf in the HSL group, compared to 207 ± 1 b·min^{-1}, at 4dpf in the LSP larvae. fH,a at 5 dpf of LSL was 218 ± 2 b·min^{-1} compared to 216 ± 2 b·min^{-1} for HSL. fH,a increased slightly to 227 ± 2 b·min^{-1} for LSL before decreasing again, while fH,a remained relatively constant for HSL. The $\dot{M}O_2$,r at 2dpf of HSL was 0.09 µmol·mg^{-1}·hr^{-1}, compared to 0.03 µmol·mg^{-1}·hr^{-1} in LSL. $\dot{M}O_2$,r subsequently peaked at 0.70 µmol·mg^{-1}·hr^{-1} at 9dpf in the HSL, compared to 0.71 µmol·mg^{-1}·hr^{-1}, at 9dpf in the LSL. These values dramatically decreased before leveling

off at around 0.20 μmol·mg^{-1}·hr^{-1} and 0.15 μmol·mg^{-1}·h^{-1}, respectively. $\dot{M}O_{2,a}$ at 5dpf for HSL was 0.38 μmol·mg^{-1}·hr^{-1}, compared to 0.57 μmol·mg^{-1}·hr^{-1} for LSL. $\dot{M}O_{2,a}$ subsequently peaked at 0.97 μmol·mg^{-1}·hr^{-1} at 10dpf in HSL, compared to 1.19 μmol·mg^{-1}·hr^{-1} at 7dpf in LSL. These values also dramatically decreased and leveled off. Significant differences ($p < 0.05$) in heart rate and oxygen consumption persisted through 21dpf. The onset of differences observed in routine and active heart rate in early larvae, correlated with parent stamina, show that juvenile or adult features are not required as a precondition for the emergence of phenotypic physiological differences.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Fish experience a multitude of environmental factors during their lifetime (see Gampler and Farrell, 2004; Pelster, 2004; Pelster, 2002). Many of these factors, such as water current speed, oxygen content and the ability of fish to adapt to hypoxic conditions play a major role in fish energetics. Larval fish have a unique physiology and ecology; therefore, they are subjected to different selective pressures than adult fish (Bagatto et al., 2001; Hunt von Herbing, 2006). For example, larval fish experience more pronounced supply-demand relationships for O_2 and CO_2 during the course of embryonic and larval development than they do as adults (Rombough, 1988). This is evident in the fact that larval fish have to expend more energy to move around in an environment that is 840 times as dense and 60 times more viscous as air, than do adults (Graham, 2006). Larval fish are small and swim much slower than adults, a situation that suggests that viscous forces have a relatively large affect at this time. The Reynolds number, which indicates the ratio of inertial to viscous forces in moving fluids, gives an index of the ease at which fish swim during different life stages; as the fish increase in age, so does the ratio (Lauder, 2006). These more pronounced supply-demand relationships have a large impact on organ systems related to gas exchange at early ages in larval fish, namely the skin and cardiovascular system.

Swimming and endurance training in adult teleost fish have been intensively studied (see Claireaux et al., 2005, Farrell et al., 1998), and some studies have examined locomotion in larval fish (see Bagatto et al., 2001; Pelster et al., 2003). However, very few if any investigators have attempted to link swimming performance in

adult fishes to the swimming performance of their offspring. Physiological performance in offspring can result from both inheritance and maternal (epigenetic) effects. My study focuses on performance in larval fishes and the extent to which the parents' swimming ability in swim tests is reflected in the physiological performance of their offspring. This study will thus attempt to generate a better understanding of transgenerational transfer of swimming performance in teleosts (specifically, the zebrafish, *Danio rerio*), and also reveal at what point in development differences in swimming ability, heart rate (routine and maximal), and oxygen consumption (routine and maximal), between low and high performance swimmers can be determined.

Before indicating the specific hypothesis I tested, let us first review the pertinent literature.

Zebrafish as a Model System for Development

The zebrafish (*Danio rerio*) is a freshwater, tropical fish native to the Ganges River in India (Axelrod et al., 1971) and also the mountains of south Asia (Laale, 1977). Zebrafish are a relatively new addition to the arsenal of model organisms available for experimental biology and are considered by many to be the ultimate "master of mutations" (Roush, 1996). Twenty years ago George Streisinger recognized the benefits of using the zebrafish to study vertebrate morphology due to its use of external fertilization and the transparency of its eggs (Detrich et al., 1999).

The zebrafish is widely used in developmental studies due to its short generation time (2-3 months), high fecundity (100-200 eggs/mating), and because the transparency of the embryos makes it easy to observe the cardiovascular system during the early developmental stages (Detrich et al., 1999). Many mutants of the wild-type (WT)

zebrafish have been made, including Albino mutants with no pigment as well as Brass mutants with little to no coloration. The lack of pigment in these two varieties enhances the view of the cardiovascular system during developmental studies which can go on longer (~21 days) than in the WT strain (~14 days).

The development of numerous cardiac and metabolic mutants (i.e., Tinman and Breakdance) (Kopp et al., 2005; Chin and Fishman, 1996) makes the zebrafish an ideal system to be used in swim studies and the effects exercise training may have on cardiac and metabolic parameters. Zebrafish also make an excellent model to study swim performance/stamina due to them being native to an area that experiences rapid water currents (Laale, 1977). The current study may be relevant to future mutant screening studies, looking at how swimming performance may affect reproductive fitness. Additionally, the well characterized genetics and intense biomedical interest in human organ function and disease make the zebrafish a good model for predicting human biology (Thisse and Zon, 2002).

Ontogeny of the Cardiovascular System in Zebrafish

Zebrafish are an ideal model for studying the development of the cardiovascular system due to the transparency of the embryos, which allows variables such as heart rate, stroke volume, and cardiac output to be calculated (Bagatto and Burggren, 2006; Hou and Burggren, 1985; Jacob et al., 2002). During development, the circulatory system of vertebrates typically starts operating earlier than any other organ (figure 1.1) (Burggren and Pinder, 1991; Pelster, 2002). As such, the heart is the first functioning organ in the vertebrate embryo. In adults the cardiovascular system is used for delivering nutrients to the tissues via convective oxygen transport. The cardiovascular

system first appears when needs for O_2 and nutrition cannot be met by diffusion alone, because of the embryos increasing volume or metabolic rate (Burggren and Pinder, 1991; Pelster and Burggren, 1996; Burggren, 2005). However, during early development even though the heart is functioning, it is not essential for survival. Zebrafish mutants have even been created that lack a heart, yet live for some period of time, to show that the cardiovascular system is not needed for survival during early developmental stages up to 5 dpf (Stainier and Fishman, 1992; Stainier et al., 1996). The purpose of the early onset of the embryonic heart has been studied and current research points toward the early embryonic heart being used in further development and maturation of the cardiovascular system; for example, increasing vascular density in critical organ systems (Burggren, 2004). Zebrafish possess a prototypic vertebrate heart with only a single atrium and ventricle; however, the zebrafish heart, like that of all teleosts, still comprises four chambers: sinus venosus, atrium, ventricle, and bulbus arteriosus (Randall, 1970; Burggren et al., 1997). Despite its apparent simplicity, the zebrafish heart shares many structural similarities with an avian or mammalian heart, making it an excellent experimental model (Stainier et al., 1996; Weinstein and Fishman, 1996; Warren and Fishman, 1998). The adult zebrafish heart is also able to withstand increases in cardiac output that would, presumably lead to increases in swimming performance (Claireaux et al., 2005).

Ontogeny of Respiration and Metabolism

Fish undergo many respiratory and metabolic changes during their lifetime (figure 1.2), including changes in the organ systems responsible for gas exchange and transport; changes in body size, which can affect metabolic rate, and also changes in

the respiratory medium from water to air, most notably seen in amphibians during metamorphosis.

The small body size of larval fish and amphibians makes cutaneous gas exchange by diffusion adequate to bring in O_2 and remove CO_2 during early developmental stages. However, once the animal reaches a larger size there is a critical point when cutaneous gas exchange alone cannot meet the needs of the animal. At this point in ontogeny convective O_2 transport is required nutrients to tissues and organ systems. Concurrently, the larval fish must switch from diffusion through the skin to using a specialized gas exchanger (gills) to increase the surface area for delivery of O_2 (Burggren and Pinder, 1991). Unfortunately, little is known about how larval fish regulate gill ventilation.

Metabolism ($\dot{M}O_2$), the process that determines the rate of oxygen consumption, also plays a crucial role in the developing zebrafish. In contrast to mammalian embryos/fetuses, which derive their nutrients from the mother, fish must rely solely on the materials present in the egg at the time of fertilization for the first several days of development. Prior to hatching, a large proportion of the embryo's energy budget is directed to growth and biosynthesis, and there is no significant increase in oxygen consumption (Adolph, 1983). However, upon hatching there is a dramatic increase in $\dot{M}O_2$ resulting from increases in muscle activity, increases in cardiac pumping, and also increased spontaneous swimming (Davenport, 1983, DeSilva, 1986, Walsh, 1989, and Weihs, 1980).

Aerobic scope – the factoral increase of oxygen consumption – plays an important role in determining the amount and length of time a fish can withstand

increases in swimming activity. Presumably, fishes with a higher aerobic scope will out-perform fishes with lower aerobic scopes.

Swimming Endurance, the Cardiovascular System, and Metabolism

Swimming is an essential fish behavior and is one of the most important factors influencing energy turnover (Brett and Groves, 1979). Fish make an excellent model to study swimming performance due to the influence of positive rheotaxy, or the instinctive ability to swim against a current. Previous studies have focused on the effects of swim training on adult fish (see Davison, 1997; Davision and Goldspink, 1977; Kieffer, 2000; Beamish FH, 1978), and more recently the effects of training on larval zebrafish (Bagatto et al., 2001; Pelster et al., 2003; East and Magnan, 1987). These adult and larval studies have shown that training leads to increases in growth rate and food conversion efficiency.

In several salmonid and cyprinid fish species, cardiovascular plasticity induced by swim training is limited at best. Even though there is an increase in heart mass due to continuous swim training, there is no significant long-term training effect on heart rate, stroke volume, or cardiac output. The lack of a significant training effect on the physiological components of the cardiovascular system leads Pelster et al. (2003) to comment that "…in fish, plasticity of cardiac muscle with respect to exercise training is quite limited." In addition, cardiovascular performance (cardiac output) does have an effect on individual swimming ability (Claireaux et al., 2005).

Metabolic rate is also affected by swim training in fish. Trained zebrafish larvae (4 to 21 dpf) tend to have higher resting metabolic, and a significantly lower oxygen consumption during training compared to non-trained zebrafish larvae (Pelster, 2002).

This proves that exposing larval zebrafish to a sustained current, increases their physiological fitness and also increases their metabolic efficiency. In reference to individual swimming ability, athletic swimmers can achieve significantly higher active metabolic rates compared to non-athletic swimmers, which overall translates to a higher aerobic scope (Claireaux et al., 2005).

Transgenerational Transfer of Swimming Performance: Maternal Factors vs. Heredity

There are two ways that swimming performance can be transferred from generation to generation: maternal (epigenetic) effects and the transfer of heritable genes (genetic effects). Maternal effects add to the complexity of studying the physiological plasticity of an animal's phenotype by speeding or slowing the rate of evolution of a physiological characteristic as compared to when no maternal effects are present (Bernardo, 1996). Maternal effects can best be explained as "…that effect which occurs when a mother's phenotype directly affects the phenotype of her offspring…" (Arnold, 1994a, p. 36). These effects lie outside of the genotype and are therefore considered epigenetic.

Maternal effects can be separated into two classes: maternal inheritance and maternal selection (Kirkpatrick and Lande, 1989). Maternal inheritance imparts the non-Mendelian phenotypic transmission and arises when the mother affects the phenotype of her offspring due to her perceiving changes in environmental conditions. With maternal selection, the mother exerts selection on offspring that is independent of the phenotype. For example, the mother may move a clutch of eggs or actively defend the nest to increase offspring survival (Bernardo, 1996).

Maternal effects can act as a mechanism for transgenerational phenotypic plasticity. The environment, as perceived by the parents, acts as a catalyst by cuing the animals to respond and adapt to the changing environment, i.e. experiencing an increase in water current speed. These changes encountered in the environment and the changes they impart on the physiology of the parents may in turn enhance offspring fitness (Bernardo, 1996).

Conversely, heritability is a measure of the genetic contribution to phenotypic plasticity. In general, heritable genes are essential for an animal's survival and must be highly refined through evolution (Klug and Cummings, 2000). Heritability studies have been used extensively in the horseracing business to determine the best way to produce superior offspring, and have consistently shown that a parents racing performance influences offspring performance (Ekiz and Kocak, 2005; Mota et al., 2005; William and Wilson, 1991).

Objectives and Hypotheses

This study focuses on the poorly understood link between the swimming and physiological performance of adult fish and their larval offspring and specifically when the parentally-derived characteristics first appear in the larvae. One can reasonably assume that when adult fish are grouped into three categories based on swimming performance stamina (high, average, and low stamina), the low stamina swimmers will have a lower cardiovascular and aerobic performance than the high stamina fish. Since many biochemical and physiological traits are inherited, offspring of low stamina swimmers should have lower cardiovascular and aerobic performance and swimming endurance than the same age offspring from high stamina swimmers.

I will test two hypotheses:

1.) The parents' ability to perform in swim stamina tests will influence the cardiac and metabolic performance of their offspring.

2.) The onset of inherited superior swimming performance, evident through differences in resting and active oxygen consumption and heart rate, appears early on in larval development before attainment of the adult morphology.

Testing these hypotheses will lead to a better understanding of the developmental physiology of zebrafish larvae, the role that parents play in the physiological performance of their offspring, and finally, at what point in development inherited differences can be observed in offspring swimming performance.

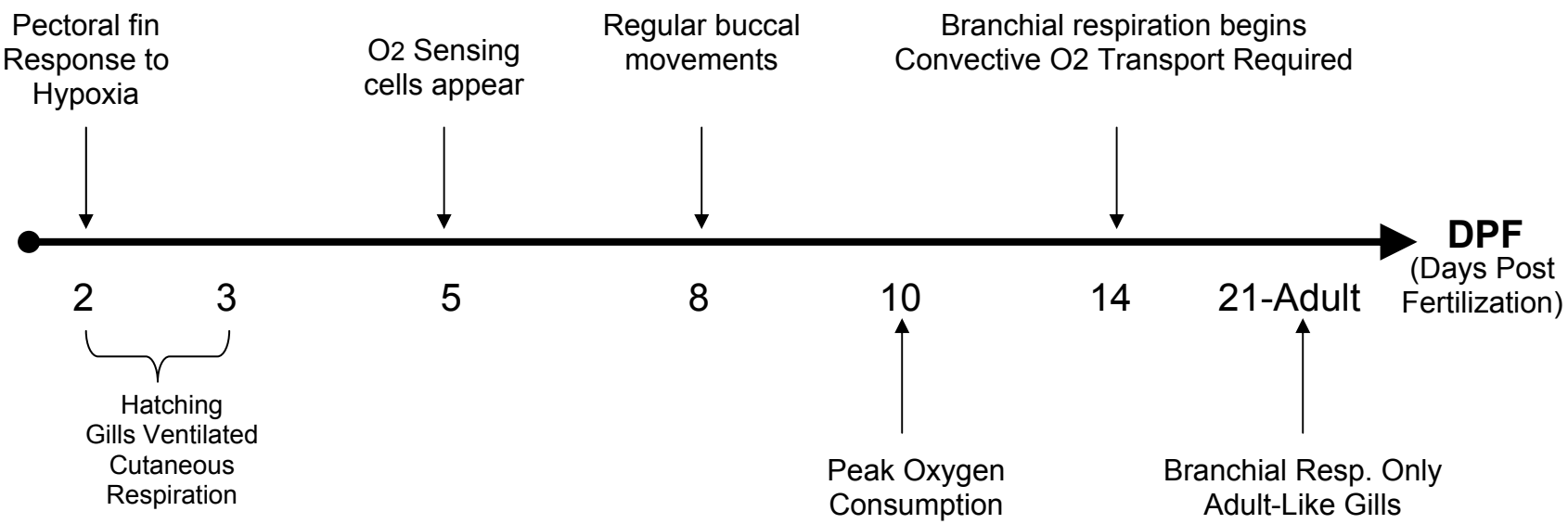

Figure 1.1 Time-line for respiratory development in zebrafish (Jonz and Nurse, 2006).

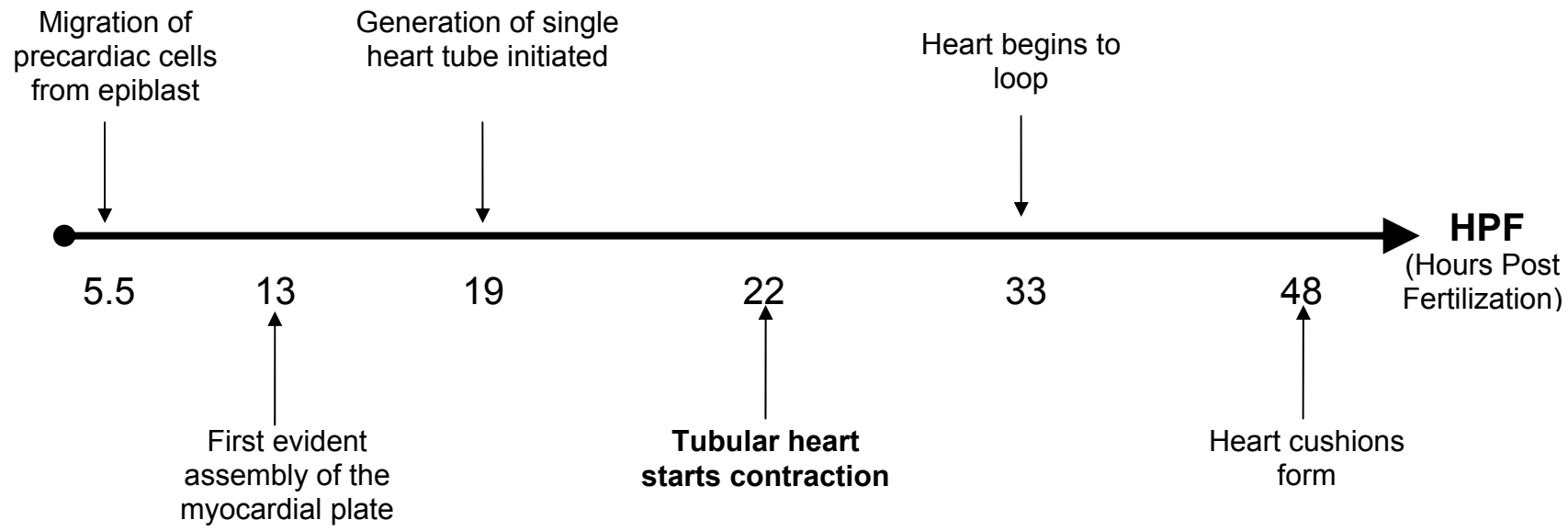

Figure 1.2 Time-line for cardiac development in zebrafish (Fishman and Chien, 1997).

CHAPTER 2

MATERIALS AND METHODS

Source of Experimental Animals

Adult zebrafish acquired from local suppliers (University of North Texas zebrafish colony, Dallas North Aquarium) were maintained in standard fish tanks (18.9 Liters). Mass, length, and width (taken behind the pectoral fin) of each fish was recorded prior to selection for further experimentation. Condition factor (K), a measure of a fishes girth (Reid et al., 2005), for these fish was also calculated by using the formula:

$$K = 100 \cdot BW \cdot TL^{-3}$$

where BW stands for body weight (g) and TL stands for total length (cm).

Swimming Protocol and Stamina Evaluation

Adult fish, selected from the general stock at random, were classified according to their swimming ability into one of three equally sized groups: low stamina swimmers, average stamina swimmers, and high stamina swimmers. Stamina was determined in a gravity fed "water treadmill" system providing fine control of water velocity (figure 2.1) (Bagatto et al., 2001). The treadmill consisted of a series of polyvinylchloride (PVC) swimming tubes that drained into a bottom reservoir where the water was then returned via a pump to a top reservoir. Each swimming tube had an inside diameter of 4 cm and a length of 45 cm, resulting in a final volume of 180 ml. Tubes were open along the length of the top to allow access to fish swimming within. Clusters of drinking straws of different lengths were cut and placed up-stream (6 cm) and down-stream (10cm) in the swimming tube to maintain laminar flow and to define the front and back boundaries of a swimming area 20 cm in length. A filtration system inside one of the reservoirs helped

maintain the correct water chemistry and filter out large particulate matter. Filtered, aerated water was continuously delivered to all tanks. Temperatures for the tanks and swim training chamber were maintained at 26-27°C for the duration of the experiment.

To separate adult fish by swimming stamina, six fish were placed in pairs in three swim tubes with a uniform current velocity of approximately 7.5 BL/s (~50% of zebrafish U_{crit} as reported by Plaut and Gordon, 1994). Water velocity was determined by measuring the time it took for dye to travel a predetermined distance. The first two zebrafish to become physically exhausted (<10 min), evident when they were swept back against the rear barrier and unable to swim forward off of it, were classified as "low stamina" swimmers. The next two fish to become exhausted were classified as average swimmers (~10-20 min), and finally, the last two fish to become exhausted were classified as "high stamina" swimmers (>20 min). After swimming stamina was determined, the fish were kept separated by stamina class in tanks with still water.

Breeding Protocol

After swimming stamina was determined, adult zebrafish from the low stamina parents (LSP) and high stamina parents (HSP) were bred within their respective group beginning one week after swimming stamina was determined. Individual breeding tanks with controlled temperature and light conditions were set up with a ratio of 2 females to 2 males. Adults were placed in the breeding tanks in the late afternoon, where they remained overnight. The next morning fertilized eggs were collected from the breeding tanks using a disposable plastic pipette, and were transferred into individual containers labeled for each group.

Growth Rates

The initial weight and length of immature fish (larvae and juveniles) was recorded at 2 and 21 days post fertilization (dpf) to assess differences in the rate of growth between performance groups. Ricker's (1979) formula for calculating weight specific growth rates was used:

$$GR = 100(W_2 - W_1) / .5(W_1 + W_2) / T$$

where W_1 and W_2 are the initial and final weights (mg) recorded over the time (T) in days, and GR is the growth rate represented as a percentage.

Heart Rate Measurements in Larvae

The protocol for swimming larvae consisted of placing the HSL and LSL in still water (0 BL/s) and then increasing the water current speed to ~ 4.5 Bl/s. Each fish then was allowed to swim continuously for 2 min. At the end of the 2 min swimming period, larvae were removed and heart rate was immediately assessed, as described below. All larvae were fed a diet of brine shrimp during resting periods.

1.) Routine Heart Rate (f_H)

Heart rate was measured in larvae beginning at 3 dpf and continued on 5, 7, 10, 14, and 21 dpf. Larvae were partially restrained for heart rate measurement by placing them in a 100µl glass capillary tube (figure 2.3) using a disposable plastic pipette. Each larva in the tube was then immediately placed on a temperature controlled (27°C) stage of an inverted microscope for heart rate analysis. Images of the beating heart were recorded with a Nikon camera, stored on a computer, and then analyzed with ImagePro Plus software. Heart rate was determined by counting the number of heart beats in a 15 sec period. The entire period of time in the capillary tube was <60 sec, decreasing the chances of significant changes in water temperature or PO_2.

2.) Active Heart Rate ($f_H a$)

Active heart rate was measured in larvae beginning at 5 dpf and continued on 7, 10, 14, and 21 dpf. Active heart rate was determined by swimming each larva in a gravity-fed swim system that was essentially a miniaturized version of that used for swimming adults. This system consisted of a 3.6 liter reservoir, acrylic tubing, and a swim chamber with an inside diameter of 1 cm and 3 cm long, resulting in a volume of 3 ml. Water temperature (27°C) and volume were maintained throughout swim training periods, and flow rate was controlled using a three-way stopcock. Larvae were allowed to swim for 2 min and then removed using a disposable plastic pipette. Once removed from the swimming apparatus by using a disposable pipette, the larvae were carefully transferred to a 100 µl capillary tube which was placed on a temperature controlled (27°C), inverted microscope stage, and heart rate was determined as described above for resting heart rate. Total time elapsed from end of the swimming bout to the end of the heart rate determination was less than 25 sec. (If greater than 10 sec elapsed following larval removal from the swim chamber, the larvae were placed back in the swim chamber for 2 min).

Oxygen Consumption Measurements

1.) Routine Oxygen Consumption ($\dot{M}O_{2,}r$)

Routine oxygen consumption ($\dot{M}O_{2,}$ r) was determined for larvae and juveniles using closed system respirometry. The respirometers consisted of 2.0 ml glass syringes with a needle and sealed with a rubber stopper. Measurements for all respirometry were done using a flow-through microelectrode (Microelectrodes, Inc., Bedford, NH), running directly into a computer chart program. The larvae were placed

in 1ml of water inside the syringes and were kept in a temperature controlled (27°C) water bath (Forma Scientific, model 2006) for one hour before measurements were taken. Oxygen consumption was calculated using the formula:

$$\dot{M}O_2\ (\mu mol\ O_2 \cdot mg^{-1} \cdot hr^{-1}) = \frac{\Delta pO_2 \cdot \alpha \cdot V}{\Delta t \cdot m}$$

where ΔpO_2 is the decrease in partial pressure of O_2 in the respirometer, α ($\mu mol \cdot L^{-1} \cdot torr^{-1}$) is the O_2 capacitance of water at 27°C, V is the initial volume in the respirometer, Δt is the elapsed time, and m is larval dry mass.

2.) Active Oxygen Consumption ($\dot{M}O_2$,a)

Active oxygen consumption ($\dot{M}o_2$,a), the oxygen consumption evident during swimming at a constant velocity of ~4.5 BL/s, was determined in larvae by using modified closed respirometry – essentially, a miniaturized Brett swimming respirometer (Beamish, 1979). Each swim respirometer consisted of glass tubing (5 mm inside diameter) shaped into a rectangle with two sample ports, a glass 2-way stopcock, and a small magnetic pump, all in series (see Bagatto et al., 2001), (Fig. 2.2). Larvae swam initially at 0 BL/s with the velocity increasing to 5 BL/s for one to two hours, depending on age (Table 2.1), before water was sampled for the partial pressure of oxygen (pO_2). $\dot{M}O_2$,a was measured beginning at 5 dpf and continued to the juvenile form.

Cost of Transport and Aerobic Scope

1.) Cost of Transport

Cost of transport (COT), a measure of swimming efficiency, was calculated using $\dot{M}O_2$,a and distance traveled by each larvae. Distance was calculated from three variables: larval body length (cm), water current velocity (cm/hr), and the amount of

time (hr) spent in the swim respirometer. COT was calculated for all measurements of $\dot{M}O_2$,a from 5 to 21 dpf. The formula used was:

$$COT=\dot{M}O_2,a \cdot (v \cdot \Delta T)^{-1}$$

Where v stands for velocity and ΔT stands for the change in time.

2.). Aerobic Scope

Aerobic scope was determined for larval fish by calculating the factorial increase in $\dot{M}O_2$,a over $\dot{M}O_2$,r, the resulting value has no units.

Statistical Analyses

Comparison of low stamina and high stamina adults were performed using a one-way ANOVA, followed by a post-hoc test to determine pairwise differences. Parametric Two-way ANOVAs were used to assess the statistical differences between high stamina and low stamina swimmers' offspring lengths, masses, heart rates, metabolic rates, and cost of transport (unless otherwise noted). If the two-way ANOVA was significant, then post-hoc tests were used to make pair-wise comparisons. All variables are represented as mean ± 1 s.e. Statistical significance is represented as having a probability less than 0.05 ($P < 0.05$). All statistical analyses were performed using SigmaStat® and SigmaPlot® software.

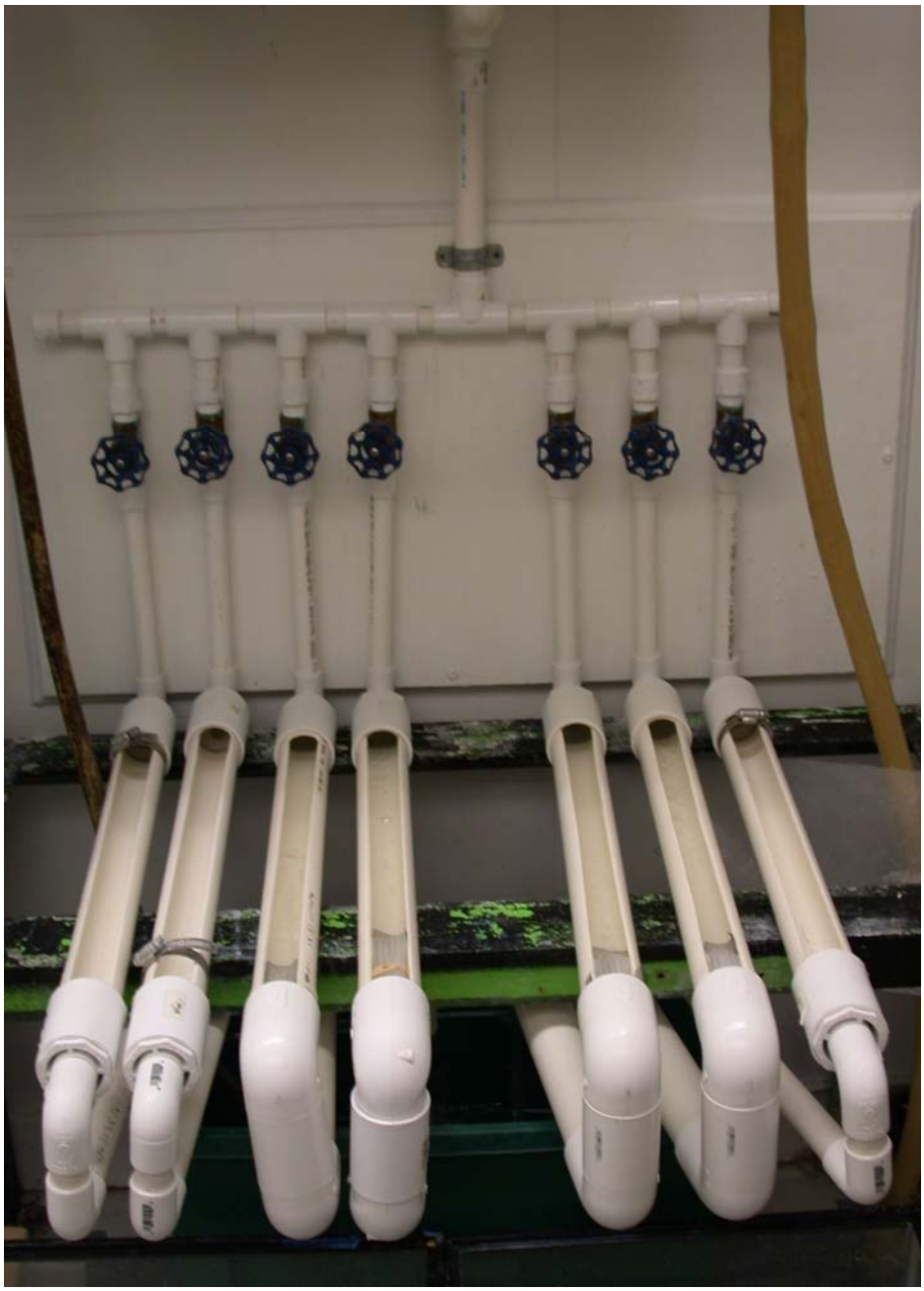

Figure 2.1 Gravity fed swim apparatus used for grouping adult zebrafish.

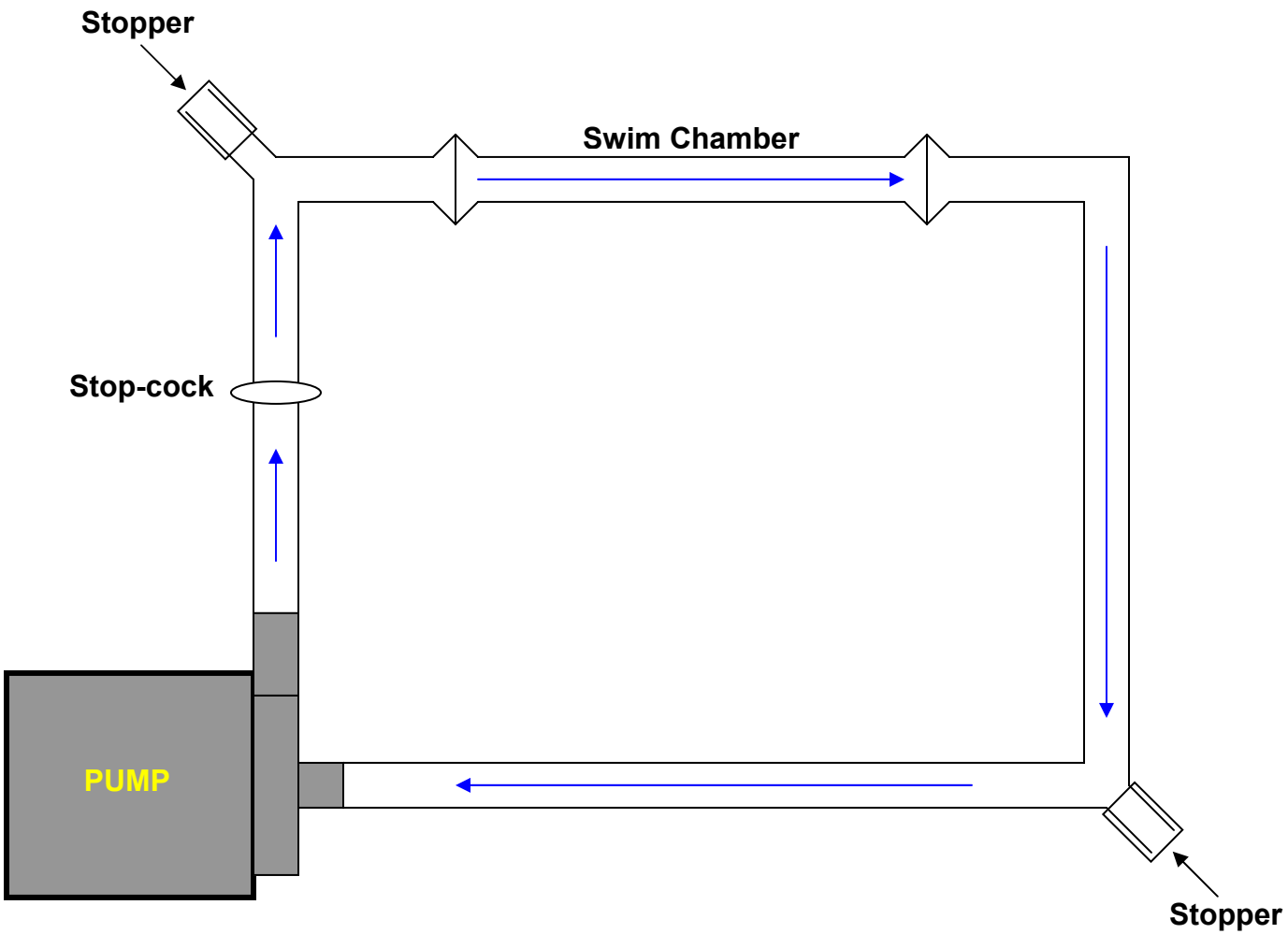

Figure 2.2 Swim respirometer used for measuring active oxygen consumption in larval zebrafish. Arrows indicate direction of water flow.

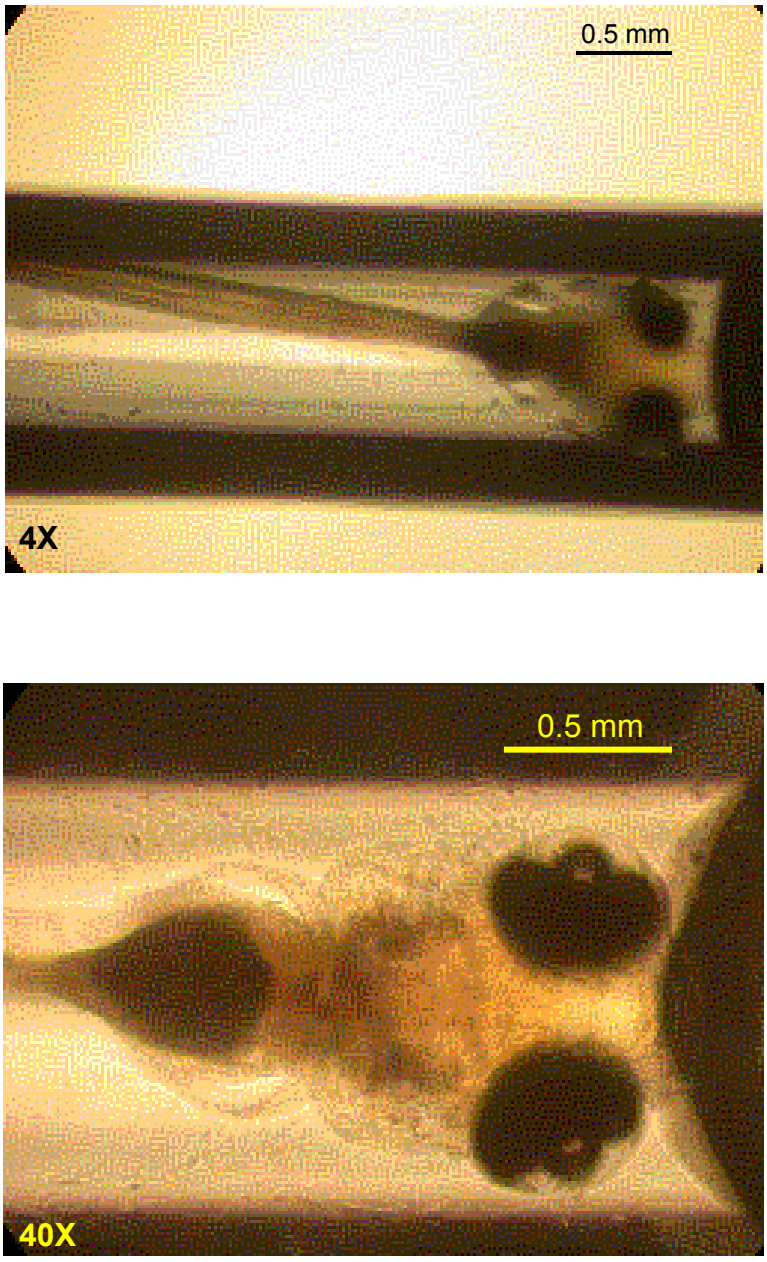

Figure 2.3 Larval zebrafish restrained in 100 µl capillary tubes for heart rate measurements at 14dpf.

Chronological Age (dpf)	Total Time in Respirometer (min)	Number of Fish per Respirometer
5	120	10
7	120	5
10	90	5
14	90	5
21	60	5

Table 2.1 Respirometry measurement variables for active oxygen consumption ($\dot{M}O_2$,a).

CHAPTER 3

RESULTS

Adult Morphometrics

Morphometrics (mass, width, length, and condition factor) for adult fish are shown in table 3.1. All values for adult males were significantly lower than females for all measurements taken, within and between groups ($P<0.05$). Adult male fish classified as low stamina swimmers had significantly shorter bodies ($P<0.01$) than fish that were classified as being high stamina swimmers, but there were no significant differences in body length in females with low and high stamina ($P>0.05$). Body masses in high stamina males and females were not significantly different ($P>0.05$) from low stamina males and females; only males showed a significant difference ($P<0.05$) in body width. Morphometric values for adult zebrafish are shown in table 3.1.

Growth Rate

Growth rate of larvae from high stamina parents and low stamina parents were analyzed separately after 21 dpf by using Ricker's formula for weight specific growth (Ricker, 1979). High stamina (HSL) and low stamina (LSL) larvae had identical initial body masses of 0.050±0.001 grams at 5 dpf, and a body mass of 0.13±0.01 and 0.12±0.01 grams, respectively, at 21 dpf (figure 3.3a). Growth rates were nearly identical in HSL (5.44%) and LSL (5.41%). Larval growth was not significantly affected by stamina group ($P>0.05$), but developmental stage did have a significant affect on body mass ($P<0.001$). Significant differences existed at 7, 10, and 14 dpf ($P<0.05$).

Initial body length at 2 dpf was 3.30±0.03 and 3.10±0.06 cm in HSL and LSL, while lengths at 21 dpf were 4.90±0.13 and 5.20±0.13 cm in HSL and LSL, respectively (figure 3.3b). Larval growth was significantly affected by developmental stage in HSL and LSL ($P<0.001$), but not by stamina group ($P>0.05$). Significant differences were present between HSL and LSL at 2, 14, and 21 dpf ($P<0.05$).

Routine and Active Heart Rate

Routine heart rates differed significantly (two-way ANOVA on ranks, $P<0.05$) between HSL and LSL (figure 3.5). LSL had significantly higher routine heart rates at 2, 3, and 21 dpf, while the HSL's routine heart rates were significantly higher at 7 dpf (Tukey's test on ranked data, $P<0.001$). Active heart rates obtained from HSL and LSL (figure 3.4b, n=8-20) were also significantly different (two-way ANOVA on ranks, $P<0.05$) and the LSL showed significantly higher active heart rates at 10 and 14 dpf (Tukey's test on ranked data, $P<0.05$ and $P<0.001$).

There were also significant differences present within the HSL for routine heart rate versus active heart rate ($P<0.001$). All developmental stages within these groups showed significantly higher active heart rates than routine heart rates ($P<0.001$ for all stages). The LSL also showed significantly higher active heart rates ($P<0.001$) and were significantly higher during all developmental stages ($P<0.001$ for all stages).

Routine and Active Oxygen Consumption

$\dot{M}O_2,r$ in HSL and LSL was significantly different ($P<0.05$) (figure 3.6). $\dot{M}O_2,r$ was significantly lower in the LSL at 7 and 21 dpf than the HSL ($P<0.05$) and was significantly higher at 10 dpf ($P<0.05$). $\dot{M}O_2,a$ in the LSL compared to the HSL was not significantly different ($P>0.10$). $\dot{M}O_2,a$ of the LSL was significantly higher than the HSL

at 7 dpf ($P<0.05$). Differences in MO_2,r and MO_2,a within groups were also highly significant ($P<0.001$), with the MO_2,a being higher at all developmental stages.

Cost of Transport

HSL and LSL showed no significant differences ($P>0.1$) in cost of transport from 5 dpf to their transition to juveniles at 21 dpf (figure 3.7). Individually, the cost of transport during development from 5 dpf to 21 dpf increased significantly ($P<0.001$) by 282% in the HSL and only 156% in the LSL. Mass-specific cost of transport was not significantly different between the two groups ($P>0.1$), and only showed specific differences at 7 and 10 dpf ($P<0.05$ and $P<0.001$) (figure 3.8).

Aerobic Scope

Factorial aerobic scope (figure 3.9) in HSL was initially 3.28 at 5 dpf and increased to 5.10 at 10 dpf before decreasing again. In LSL, aerobic scope was initially 6.0 at 5 dpf and decreased to 1.2 at 14 dpf before increasing again. The aerobic scopes of LSL were 82%, 109%, and 139% higher than HSL at 5, 7, and 21 dpf, respectively. Aerobic scopes of HSL were 102% and 18% higher than LSL at 10 and 14 dpf, respectively.

Group	Length (cm)	Width (cm)	Mass (g)	Condition Factor (K) ($100*g*cm^{-3}$)
HSP – Male	$3.23 \pm 0.03^{a,b}$	0.31 ± 0.01^{a}	0.22 ± 0.01^{a}	0.63 ± 0.03^{a}
HSP – Female	3.36 ± 0.02	0.35 ± 0.03	0.31 ± 0.02	0.87 ± 0.07
AVG – Male	3.22 ± 0.05^{a}	0.26 ± 0.01^{a}	0.20 ± 0.01^{a}	0.60 ± 0.02^{a}
AVG – Female	3.29 ± 0.06	0.31 ± 0.01	0.26 ± 0.01	0.73 ± 0.03
LSP – Male	3.05 ± 0.06^{a}	0.25 ± 0.01^{a}	0.18 ± 0.02^{a}	0.51 ± 0.06^{a}
LSP – Female	3.46 ± 0.05	0.34 ± 0.02	0.34 ± 0.03	0.95 ± 0.07

Table 3.1 Adult morphometrics in male and female zebrafish. Values are means ± 1 s.e. a=significantly different than female of same group, b=significantly different than LSP male.

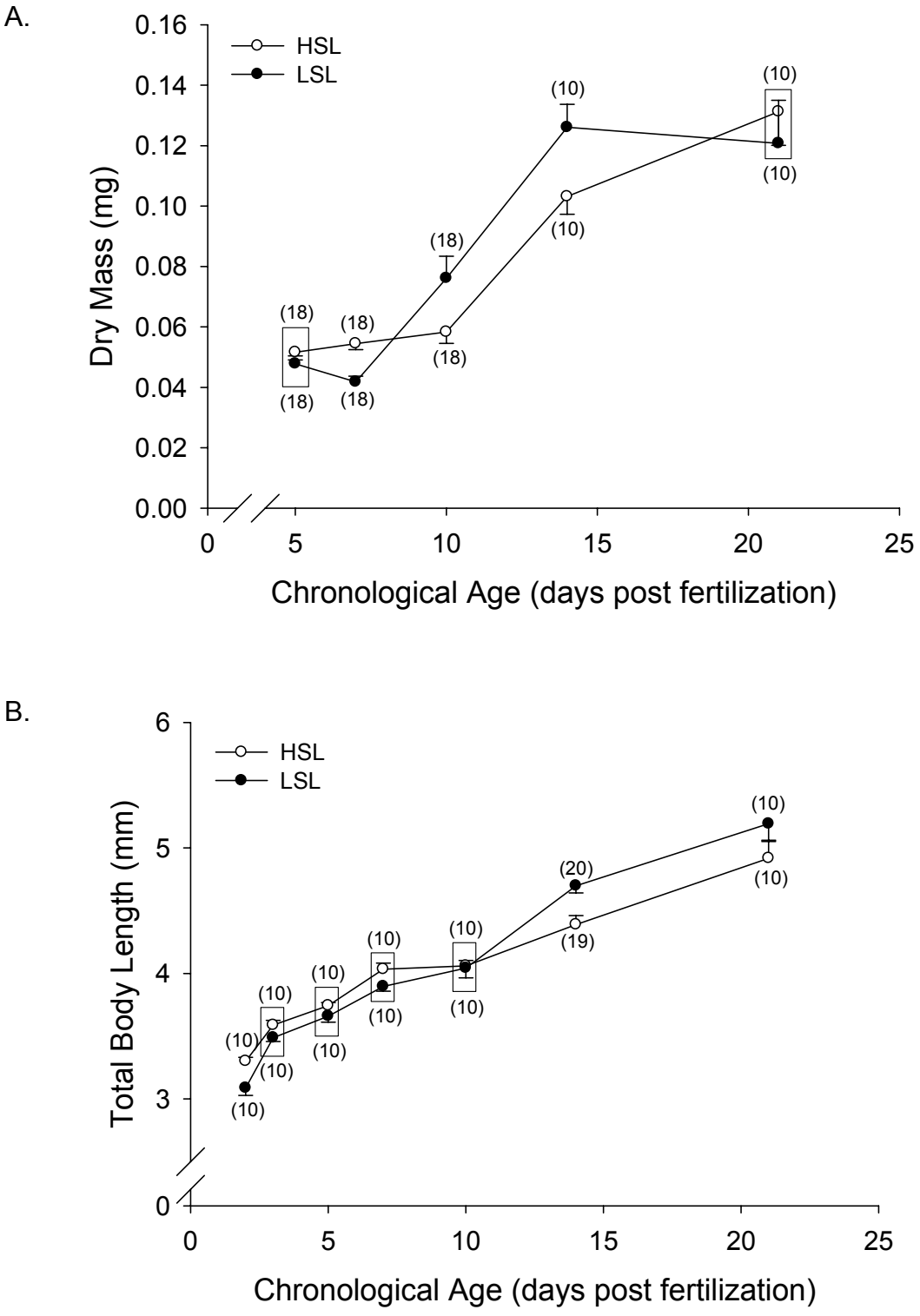

Figure 3.1 HSL and LSL dry mass (A) and total body lengths (B) taken during different stages of development. Values are means ± 1 s.e., boxes represent values that are not significantly different.

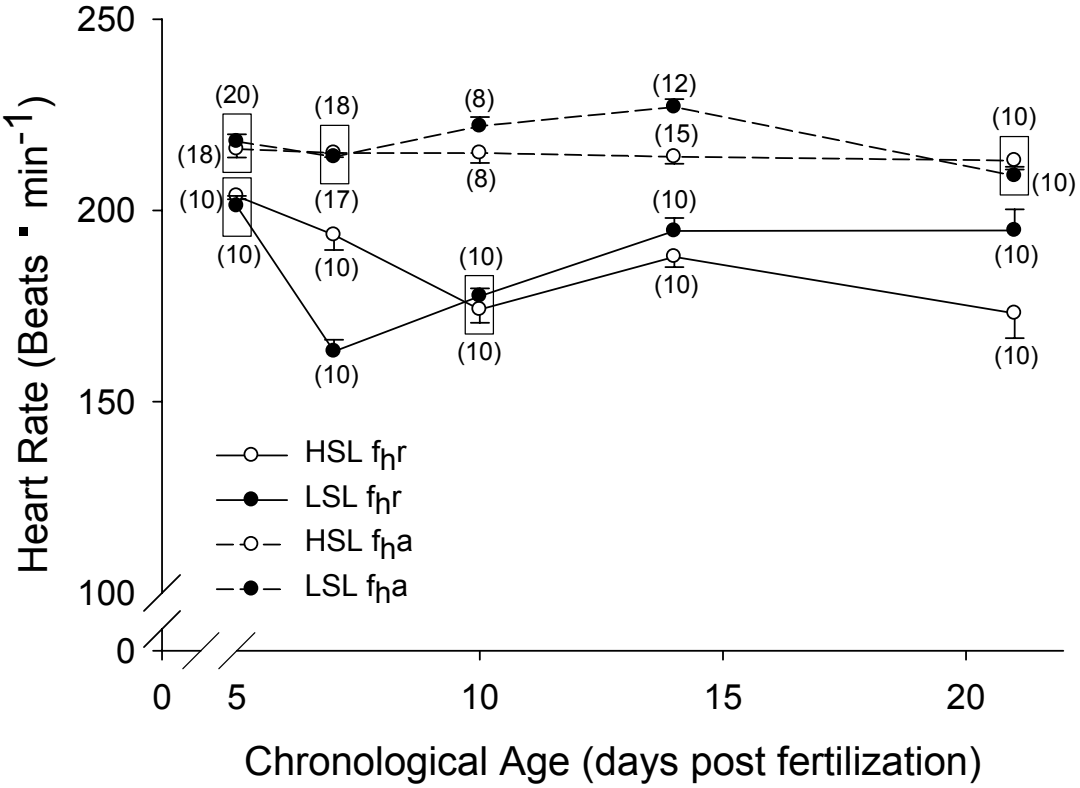

Figure 3.2 f_H and f_Ha of HSL and LSL zebrafish groups taken during different stages of development. Values are means ± s.e., boxes represent values that are not significantly different.

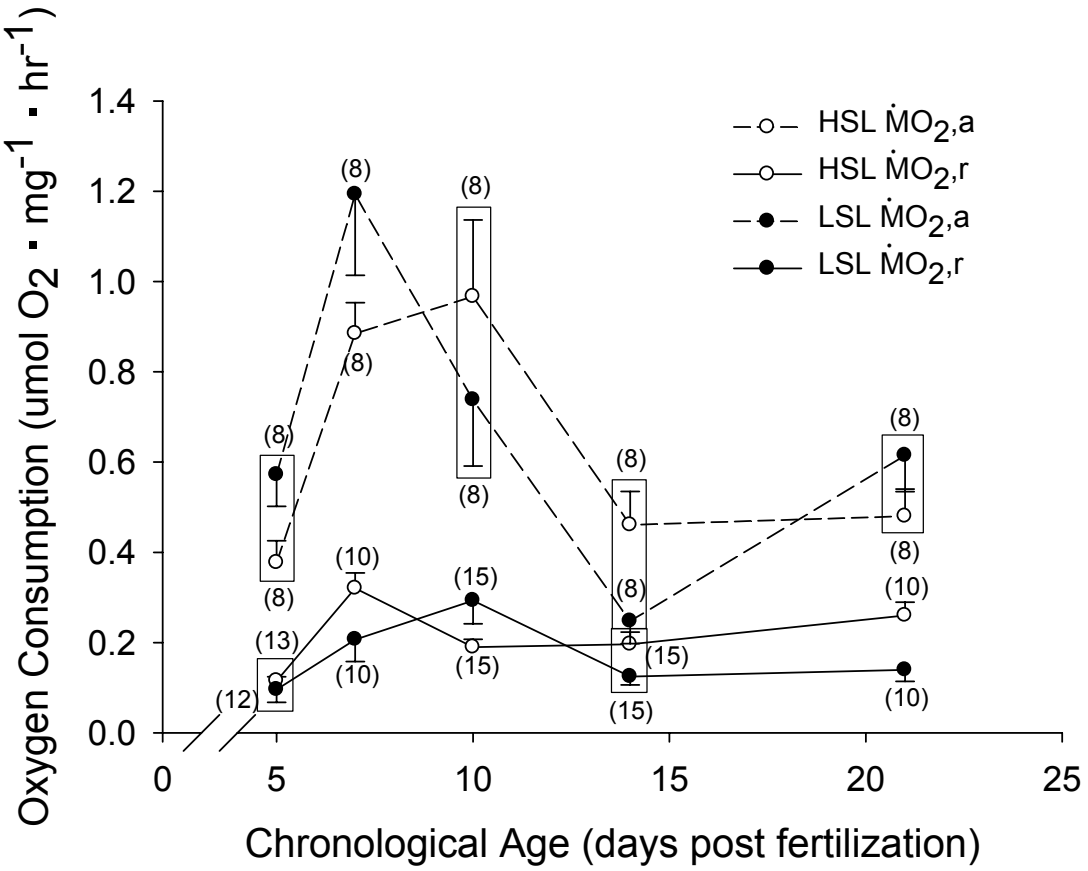

Figure 3.3 $\dot{M}O_2$,r and $\dot{M}O_2$,a of HSL and LSL groups taken at different developmental stages. Values are means ± 1 s.e, boxes represent values that are not significantly different.

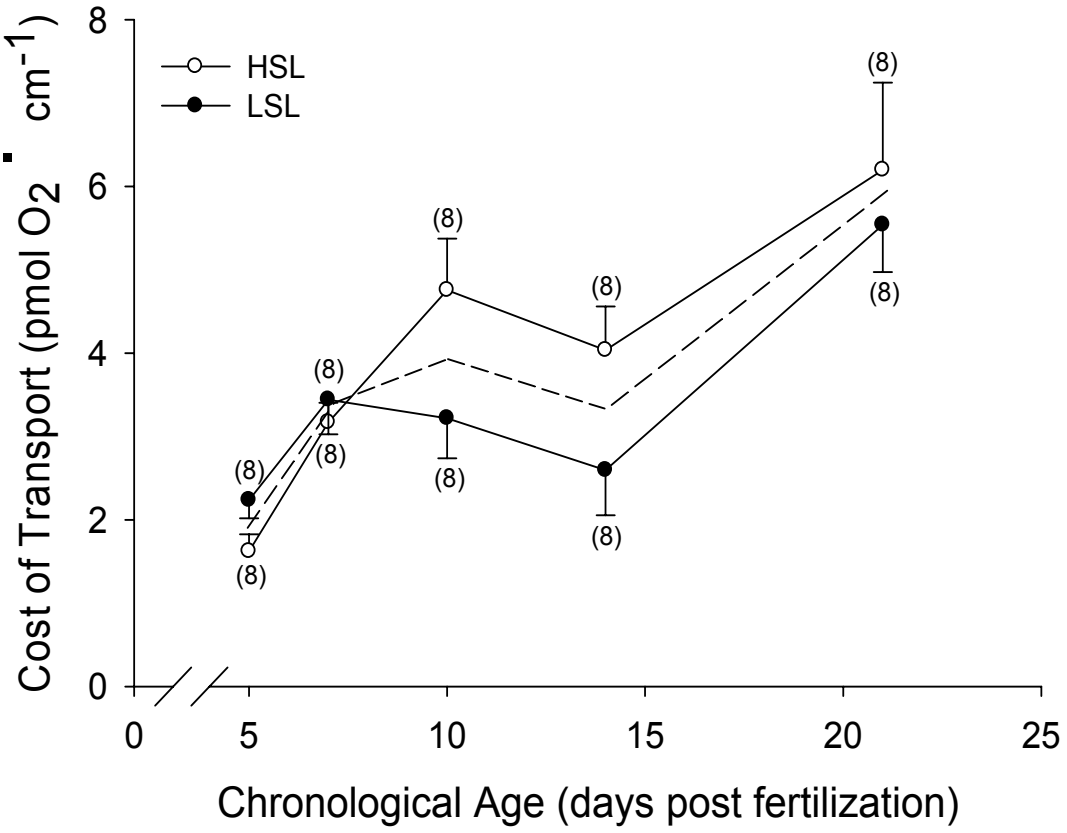

Figure 3.4 Cost of Transport (COT) in HSL and LSL groups taken at different developmental stages. Values are means ± 1 s.e., the dashed line represents the mean of HSL and LSL taken together.

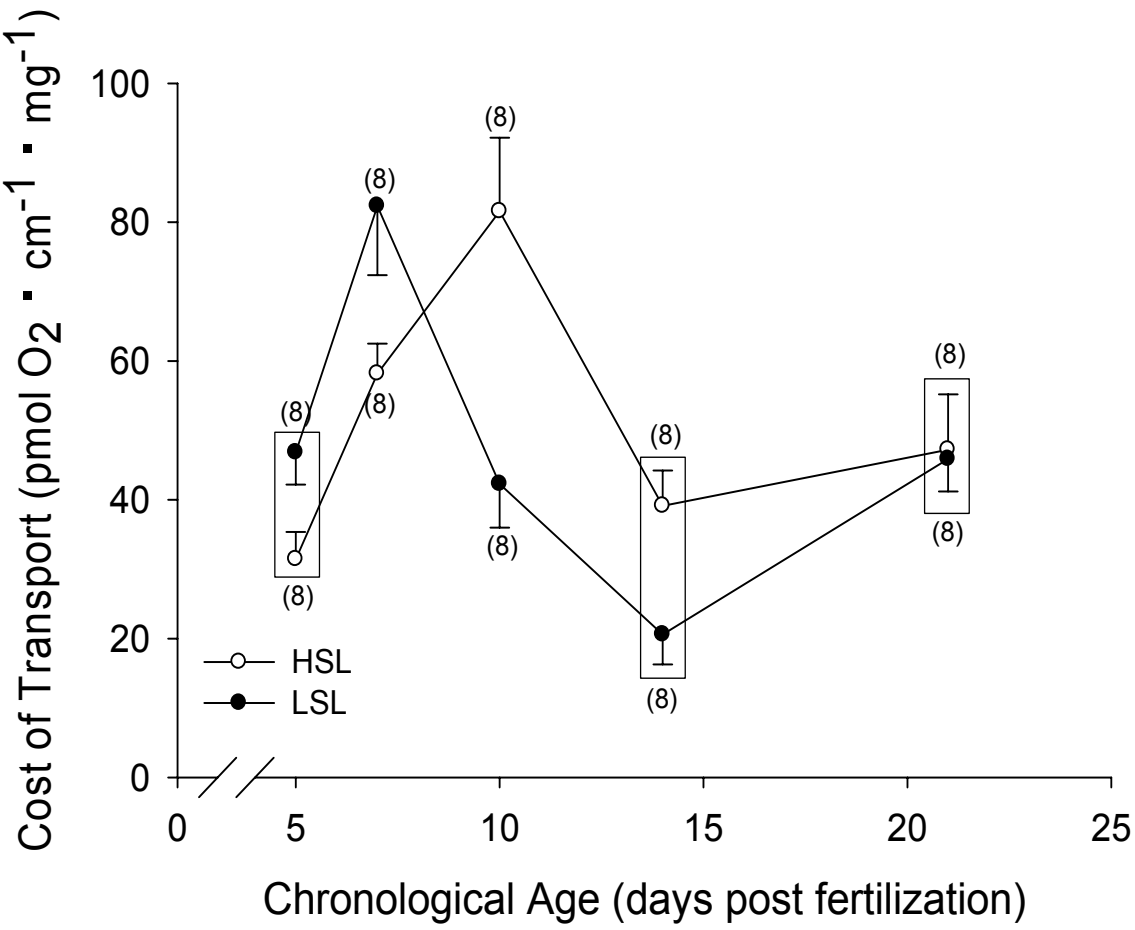

Figure 3.5 Mass-specific cost of transport in HSL and LSL groups taken at different stages of development. Values are means ± 1 s.e, boxes represent values that are not significantly different.

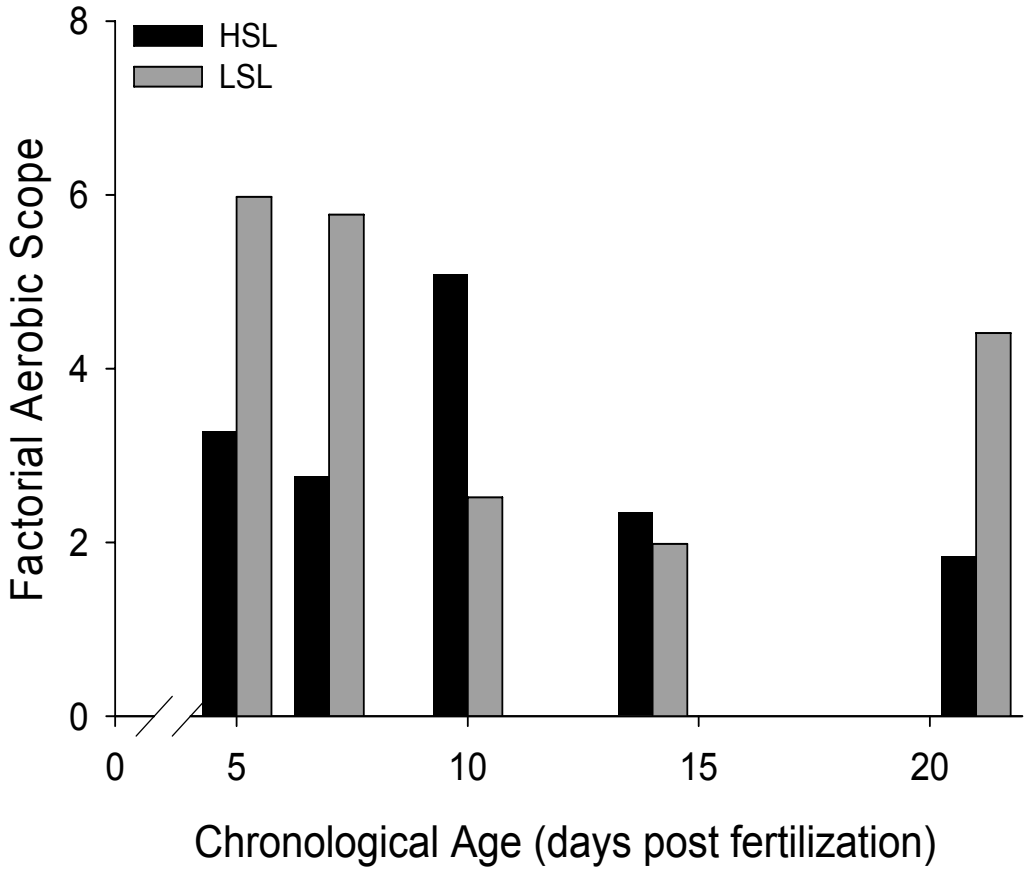

Figure 3.6 Factorial aerobic scope of high stamina and low stamina larval zebrafish.

CHAPTER 4

DISCUSSION

Adult Morphometrics and Swimming Stamina

Body shape plays a major role in fish hydrodynamics (McHenry and Lauder, 2006). However, body morphology generally did not play a significant role in the swimming stamina of adult zebrafish in this study. The only significant difference was in the greater length of the high stamina males when they were compared with other groups. As zebrafish grow toward the adult morphology, they are better able to cope with the viscous forces of water by making their bodies more streamlined compared to the larval or juvenile fish (McHenry and Lauder, 2006). This streamlining is an important factor in evolutionary changes that reduce the energetic costs of locomotion, and reduces the amount of drag the fish experiences by providing a more hydrodynamic shape (Vogel, 1981).

There are many plausible explanations for the lack of differences in adult morphology in the high stamina and low stamina adults. Certainly adults with identical gross morphology may have one or more underlying physiological differences (respiratory, cardiac). Alternatively, or in addition, the biochemistry/metabolism could be different in important ways. Finally, the distribution of white and red muscle could differ in high and low stamina adults without any outward, gross morphological differences (Davie et al., 1986). Many factors go into determining how well an organism performs (table 4.1); at slow to fast sustainable swimming speeds (the kind observed in our experiments) the main factors are motivation and maximal oxygen consumption. Maximal oxygen consumption is only as efficient as the respiratory and cardiovascular

systems supplying oxygen to the skeletal muscle. There could be numerous differences that account for the differences seen in swimming stamina in adult fish, but determining these awaits further studies.

Growth Rates

Growth throughout early development is a fundamental process shared by all animals, and is one that is necessary for reproduction and ultimately survival. Many abiotic and biotic factors influence the rate at which animals grow, and these rates can vary greatly within and between species. Abiotic factors that affect growth include the temperature, pH, salinity, and oxygen content of the surrounding environment (Arnott et al., 2006). Biotic factors that affect growth include food availability, predator/prey relationships, and maternal or genetic factors.

In this study, low stamina larvae had significantly higher dry masses at 7, 10, and 14 dpf, and had greater length by 14 and 21 dpf. Food availability and abiotic factors were not a factor in these controlled experiments, but competition for food can ultimately lead to some fish growing faster and larger than others, as seen in gilthead sea bream (*Sparus aurata*) (Goldann et al., 2003) and juvenile cod (Hart and Salvanes, 2000). Swimming performance was not measured during this study, so the effect of these length and mass differences are unknown. However, as mentioned above, the hydrodynamics of fish can be affected by their body shape.

In general, a faster growth rate would be more beneficial than a slower rate, allowing the larvae to reach an adult size more quickly. However, rapid growth brings along with it some disadvantages, namely the energetic cost required to sustain maximal growth. For example, in the Atlantic silverside fish, *Menidia menidia*, faster

growing fish incur an increase in short-term oxygen demand required for processing larger, more frequent meals (Arnott et al., 2006). However growth rate had no effect on maximal oxygen consumption, thereby decreasing the metabolic scope available for other physiological processes, such as swimming. Therefore, the fact that LSL showed higher body mass and total lengths at certain points in development than HSL, could attribute to differences seen at the same time in metabolic scope in our experiments.

Heart Rate

The onset of differences observed in routine and active heart rate in early larvae, correlated with parent stamina show that juvenile or adult features are not required as a precondition for the emergence of phenotypic physiological differences. Heart rate can be a good indicator of overall cardiac performance and cardiac output in fishes (Burggren et al., 1996). In many fishes, cardiac output is achieved primarily by an increase heart rate rather than stroke volume (e.g. yellowtail kingfish *Seriola lalandi*, Clark and Seymour, 2006). Data from the present study show that by 10 days of development there are clear differences in routine and active heart rates between high stamina and low stamina larvae. It is also worth noting that there was a significant increase in heart rate after the larvae had acute exposure to an induced water current in both groups, showing that increases in aerobic stress are observable through heart rate during early development.

The lower routine and active heart rate values for HSL might reflect an increase in stroke volume, leading to an increase in cardiac output. Trout grouped according to swimming performance and assessed 9 months later, had a maximum cardiac output that was 30% higher for the good swimmers compared to fish classified as poor

swimmers. In these experiments heart rate and stroke volume both increased significantly, but it was determined that the differences in cardiac output were mainly the cause of increase stroke volume (Claireaux et al., 2005).

Other factors that could possibly influence the efficiency of cardiac performance are heart shape and mass, and also the amount of systemic resistance present during maximum aerobic activities. A larger, more muscular, ventricle could result in an increase in stroke volume, leading to increased cardiac output, as might a decrease in vascular/systemic resistance. Either or both sets of changes would lead to more oxygen-rich blood being delivered to muscle tissues required during maximum aerobic exercise. Additional experiments are needed to tell whether the differences in heart rate in the two groups actually translate to a higher swimming stamina in older juveniles or adults.

Oxygen Consumption

The general patterns of change in oxygen consumption during early development observed in the present study are comparable to previously published data on salmonids and zebrafish (Rombough, 1988; Rombough, 1988; Rombough and Ure, 1991; Barrionuevo and Burggren, 1999). Zebrafish show a significant increase in oxygen consumption during the first 8-10 days of development, most likely due to increasing length and organogenesis, leading to an increase in metabolically active tissues. Soon after the peak in oxygen consumption at approximately 10 dpf, larval zebrafish require the use of the gills and blood for convective oxygen transport, leading to a decrease and then stabilization of aerobic metabolism (Jonz and Nurse, 2006). In addition to regulatory mechanisms being in place, the reduction of mass-specific oxygen

consumption may also be in part due to allometry because of the dramatic increase in body mass during development (Barrionuevo and Burggren, 1999).

Routine oxygen consumption in the present study, like heart rate, differed between HSL and LSL during development, with the "developmental trajectory" for routine oxygen in the low stamina larvae consumption shifted slightly to the right in a plot of $\dot{M}O_2$ as a function of development (figure 3.6). This shift could be due to the LSL undergoing either a longer period of organogenesis or a delayed conversion of egg yolk into new metabolizing biomass (Barrionuevo and Burggren, 1999). Either phenomenon could result from genetic or maternal influences. Like heart rate, the early onset of differences in oxygen consumption shows that juvenile or adult features are not a required precondition for the expression of differences related to parental characteristics.

Another key determinant in oxygen consumption is the ability of the heart to supply oxygen to aerobic muscle tissue; this is a major factor that can actually limit maximum aerobic capacity (Hussain et al., 2001). The results for active oxygen consumption show a slight delay in peak oxygen consumption during development for high stamina larvae compared to low stamina. This delay could possibly be related to these larvae being better equipped to cope with the increasing demands for oxygen delivery to the muscle tissues. Active heart rate during the same period for high stamina larvae stays relatively constant, suggesting that these larvae are coping by increasing their cardiac output by increasing their stroke volume rather than heart rate.

Cost of Transport

Cost of transport is used as a measure of swimming efficiency and to compare energetic costs incurred by locomotion. The cost of swimming increases approximately 220% during the transition from larval to juvenile zebrafish. This increase can most likely be attributed to changes in morphological characteristics that take place during this time. As body size and surface area increase during development, so does drag, leading to an increase in the energetic costs of swimming (Webb, 1975). Interestingly, despite differences in body mass and length, there was no difference in cost of transport between HSL and LSL during development.

Aerobic Scope

The level of aerobic performance in fish can dictate the extent of the aerobic scope. By 5 dpf there is a highly significant increase in oxygen consumption between routine and active measurements for both groups, suggesting that at this early developmental stage larval fish have the ability to respond to increased aerobic activity by increasing delivery of oxygen to muscle tissues. Low stamina larvae showed higher aerobic scopes on 3 of the 5 days measured during development, resulting from both higher rates of active oxygen consumption and lower routine oxygen consumption. Aerobic scope in marine teleosts is greatly affected by larval growth and developmental trajectory (Killen et al., 2007), therefore, the differences seen in aerobic scope might also relate back to the differences seen in larval mass and length.

Conclusions and Future Directions

The onset of differences in cardiac and metabolic performance appears early on in development and does not require the presence of juvenile or adult characteristics. These results also show that a parent's swimming stamina does play a small but

significant role in determining the level of cardiac and metabolic performance evident in their offspring even before these offspring attain adult morphological characteristics. It is not yet evident to what extent these differences affect the fitness of larva. However, they do reveal that the transgenerational transfer, either through inheritance or maternal effects, of characteristics related to swimming performance becomes evident very early in development. This provides yet another example in which zebrafish, with their rapid growth and short generation time, are a good model for studying physiological attributes.

Are the differences in cardiac and metabolic performance due to a genetic component (heritability), or are they due to some epigenetic factor such as maternal effects? To solve the heritability question, offspring from both groups of parents will need to be placed in the same gravity-fed system and have their swimming stamina observed by measuring their time to exhaustion. To answer the maternal effects question, two groups of adults would need to be maintained: a control group, with no induced current; and an experimental group that stays in a moderate-speed, induced current environment. Both the control and experimental groups would need to be bred, and the offspring's swimming stamina recorded.

Mentioned above, cardiac output, stroke volume, and ventricle size also need to be measured. These measurements would help to determine whether cardiac output is playing a role in the aerobic performance of these fish, and if so, is this increase due to an increase in heart rate, stroke volume, or a combination of heart rate and stroke volume.

Finally, the current study could also provide an avenue for further study of the locomotor ecology, behavior, and evolution of fishes by using the zebrafish as a model system or screening tool. Recently, several fish species (e.g. Trinidad guppies and North American Sticklebacks) have been used to study patterns of natural selection and evolutionary response to predation (Lauder, 2006). One of the main parameters measured in these studies is locomotor performance and the effects it has on the fitness of fishes. Another significant area of research involves the study of migratory, anadromous fish, such as the Atlantic salmon (*Salmo salar*) and non-migratory, resident fish stocks in streams and rivers and the metabolic differences that exist. Morinville and Rasmussen (2006) have shown that the more aggressive anadromous fish possess the metabolic scope and morphological shape to persist in fast currents, while resident brook trout do not.

A major constraint that could affect the fitness of fish that reside in fast currents is the presence of man-made culverts and dams that fish cannot navigate, resulting in the loss of anadromous forms and also changes in the use of upstream habitats (Morinville and Rasmussen, 2006). Fish ladders are being constructed to bypass these man-made obstacles, providing another way to reach spawning areas and maintain fish stock numbers. Successful movement to more suitable environments increases evolutionary fitness and may depend on the locomotor capacity of the fish (Nelson et al., 2002). This study could provide a novel tool for measuring/selecting superior swimming performance; and allow for the determination of transgenerational transfer of superior swimming performance either by inheritance or maternal factors.

Speed	Limiting Factor
Slow sustainable speed	Motivation, ultimately fuel (food)
Fast sustainable speed (aerobic) -endurance	Maximal oxygen consumption
Fast non sustainable speed (anaerobic) -maximal exertion	Anaerobic metabolism
Maximal burst speed	Structure/function of the musculoskeletal complex

Table 4.1 Major factors limiting locomotor performance at different levels of exertion (from Bennett and Huey, 1990)

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